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(19)(CA) APPLICATION FOR CANADIAN PATENT (12)

(54) Recombinant Coccidia and Its Use in a Vaccine

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ABSTRACT OF THE DISCLOSURE

The present invention provides for recombinant coccidia comprising a relatively non-pathogenic strain of coccidia to which has been introduced DNA from one or more other strains or species of coccidia. In an aspect of the invention, there is provided a recombinant coccidiosis vaccine comprising a relatively non-pathogenic strain of coccidia to which has been introduced sufficient DNA of one or more other strains or species of coccidia to produce immunity to the multiple species in a host through inoculation of the host by the recombinant species.

RECOMBINANT COCCIDIA AND ITS USE IN A VACCINE

FIELD OF THE INVENTION

The present invention is directed to a recombinant coccidia in which the DNA of other strains or species of coccidia has been introduced into a relatively non-pathogenic strain of coccidia. The invention is also directed to a coccidiosis vaccine utilizing the recombinant coccidia to produce immunity to multiple species of coccidia in a host through inoculation of the host by the recombinant species.

BACKGROUND OF THE INVENTION

Coccidiosis is an intestinal disease caused as a result of infection by coccidia, an obligate intercellular protozoa. As infections are transmitted by environmentally resistant oocysts released in the feces of infected animals and because livestock are often kept together in large numbers, coccidiosis has become an important disease of livestock throughout the world. In poultry, coccidiosis is the most frequently recorded disease which according to some estimates is responsible for losses in excess of $1 billion per year. Without adequate measures to control coccidiosis, the poultry industry could not continue to exist.

In poultry, coccidiosis is a result of infection by coccidia of the genera *Eimeria*. There are at present

seven known species of *Eimeria* which infect chickens; *E.acervulina*, *E.brunetti*, *E.maxima*, *E.mitis*, *E.necatrix*, *E.praecox* and *E.tenella*. *E.tenella* and *E.necatrix* are the most pathogenic followed by *E.brunetti* and *E.maxima*. In turkeys, there are also at present seven known species with *E. adenoides* considered the most pathogenic and *E. gallopavonis* and *E. meleagrimitis* also considered important in terms of clinical disease. Eimerians are host specific in that those species which infect one species of host do not generally infect other hosts. Not only are the Eimerians host specific but they parisitize specific sites within the host and specific cell types within a tissue or organ. In chicken, species of *Eimeria* parasitize and develop in different regions of the gut with *E.acervulina* occupying the most proximal region and *E.tenella* and *F.brunetti* the most distal regions. In addition, different stages of a single species can be specific to different regions of the intestine and different cell types within that region. In *E.necatrix* infections, for example, two generations of schizogony occur within crypt epithelial cells of the ileum but gametogony occurs in surface epithelial cells of the ceca.

Species of *Eimeria* generally develop in epithelial cells of endodermal origin, with the majority parasitizing epithelial cells of the intestinal mucosa. Coccidiosis infection generally results in villus flattening or atrophy, crypt hyperplasia and decreases in villus/crypt ratios accompanied by varying degrees of malaise, diarrhea, maladsoption, and reduction in growth rate. Severe infections frequently lead to death.

As early as 1929 (Tyzzer, 1929), it was shown that immunity developed during a coccidial infection such that birds which recovered from such infections were generally immune to further infection. Studies have described the presence of numerous antigens in *Eimeria* species with different antigenic profiles associated with

the different stages of the development of the *Eimeria* (Wisher 1986; Clarke et al, 1987; Jenkins et al, 1988; Lillehoj et al, 1988; Brothers et al, 1988, Profous-Juchelba et al, 1988, Castle et al, 1991). The life stage of *Eimeria* parasites is intricate consisting of several differing generations of asexual replication followed by the development of the sexual stages. These different life cycle stages differ in immunogenicity and antigenic composition (McDougall et al (1986)). Recent work has indicated that the gametogony stages are not as immunogenic as developing asexual forms of the parasite and more recent studies have indicated that of all the stages, the sporozoite, particularly in *E.tenella*, may be the major target for the development of the immune response in the host. The nature of the immune response in the host is generally a combination of antibody mediated and cell-mediated responses with T-cell dependent immunity being the prevalent mechanism of immunity to infection (For review see Rose chapter in Long (1990)).

Coccidiosis control is most commonly accomplished through the use of anti-coccidial drugs. The anti-coccidial drugs are mixed into the feed at low levels to be given continuously to the growing chickens for continuous prevention of coccidiosis. Amongst the anti-coccidial drugs most commonly used are the polyether ionophores with monensin being the most commonly used ionophore. Monensin is usually administered in the range of 60 to 90 rpm and is continually administered to the animal from birth up to slaughter although some producers continue the practice of weaning off the monensin for 5 to 7 days prior to slaughter. Other commonly utilized anti-coccidial drugs include salinomycin, lasalocid, narasin, maduramaicin, and semduramicin, nicarbazin. By far the most significant use of anti-coccidial drugs is in broiler chickens which is estimated to cost in excess of $350 million yearly.

The intensive use of the anti-coccidial drugs has lead to the emergence of coccidials with reduced sensitivity or drug resistance. When a coccidium becomes resistant to a drug there is usually a collateral resistance to other drugs of the same chemical class but not to unrelated drugs. Drug resistance has been found to be a genetic trait and tends to remain in a population for many years. Coccidia have more difficulty in becoming resistant to some drugs than in others. For example, resistance to halofuginone, clopidol, quinolones and robenidine has been found within a year of the use while resistance to monensin and other ionophores were slower to develop but in recent years the incidence of coccidial resistance to the ionophores has increased significantly (See McDougall chapter in Long,1990).

To attempt to prevent the development of drug resistance, alternate use of drugs has been proposed using programs such as shuttle and rotation programs. A shuttle program is practiced within the growout of a flock where one drug is used for the first three to four weeks and then another drug is used for the remainder of the flock's life. Generally, in this program, a strong drug is used initially in the starter feed followed by one of the ionophores such as monensin in the grower feed. A rotation program simply alternates the use of two or more drugs at various intervals between flocks such as over three to six month periods.

The development of drug resistance has limited the effectiveness of anti-coccidials and it is likely that this development will intensify over the next few years. To overcome this, considerable effort has been spent aimed at the development of vaccines which will provide long term immunity to the animal without the necessity of utilizing anti-coccidial drugs.

Owing to the intricacy of the life stages of the coccidia and the numerous antigens present with the different antigenic profiles associated with the different stages of development, the production of suitable vaccines has not proceeded as well as had been hoped. While there have been isolated reports of immunization against a specific strain of coccidia through the use of isolated antigen (Murray et al, 1986), no one antigen or series of antigens has been developed which will provide protection against all species of coccidia which infect the animal. Such protection has only been accomplished through the use of live vaccines which contain oocysts from all relevant species of coccidia. Examples of vaccines presently commercially available include: COCCIVAC and IMMUCOX, both live, virulent vaccines containing oocysts from the seven species of *Eimeria* that parasitize domestic fowl, and PARACOX, a live, attenuated vaccine.

The vaccines are generally administered either in one large dose within the first few days of life or through the use of smaller doses given singularly or at separate times over the first few weeks of life.

However, the use of the vaccines at present have some potential problems. The use of the live, virulent vaccines such as COCCIVAC and IMMUCOX, if not properly administered could result in some infection of the flock with coccidiosis. Alternatively, if not enough of any of the presently available vaccine is administered then the flock will not attain the necessary immunity to become resistant to coccidia infection. Additionally to promote complete protection the vaccines require the combination of numerous species and strains of *Eimeria* which may present problems in the proper formulation of the vaccine.

SUMMARY OF THE INVENTION

The present invention provides for a recombinant coccidia comprising a relatively non-pathogenic strain of coccidia to which has been introduced DNA from one or more other strains or species of coccidia.

In an aspect of the invention, there is provided a recombinant coccidiosis vaccine comprising a relatively non-pathogenic strain of coccidia to which has been introduced sufficient DNA of one or more other strains or species of coccidia to produce immunity to the multiple species in a host through inoculation of the host by the recombinant species.

BRIEF DESCRIPTION OF THE DRAWINGS

A preferred embodiment of the present invention is illustrated in the attached drawings in which:

Figure 1A is a photograph of ethidium bromide stained electrophoretically separated DNA from the parent strains and the recombinant strain in agarose gel before transfer of the DNA to nylon membrane, and

Figure 1B is a photograph of X-ray film of the distribution of radioactive Yeast Artificial Chromosome (YAC) probe sequence bound to the DNA of Figure 1A after transfer to a nylon filter.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based upon the principle that genomic DNA of related species of coccidia is similar enough in genomic organization and gene expression to permit productive recombination to take place if DNA from one species were to be introduced into cells of another species at the right time.

A method for isolating genomic DNA from coccidia was worked out based on isolation methods for filamentous fungal DNA. The method basically involves the extraction of the DNA by use of a French press followed by phenol and ethanol extractions. The isolated DNA is then transferred to an recipient host coccidium by suitable means to permit the expression of the donor DNA in the recipieint host coccidium. As coccidia DNA is present in the nucleus in discrete chromosomes, the suitable means for transfer will allow the donor DNA to either integrate into the host chromosomes or will allow the donor DNA to replicate as discrete additional chromosomal material. To permit the replication of the donor DNA as discrete chromosomes, chromosome functional sequences such as suitable telomeric sequences may have to be added. Such suitable telomeric sequences may be provided by packaging the DNA in a suitable vector acting as an artificial chromosome which contains such sequences for transfer of the DNA. Several such vectors have been developed for yeast which fortuitously have telomeric sequences derived from protozoan sources such as *Tetrahymena*. The use of such vectors has the added benefit that, not only do they permit the transfer of the DNA between species of coccidia, but also enable the cloning of individual genes of the coccidia in both prokaryotes and yeast as the vector acts as a plasmid in eukaryotes such as *E. coli* and as an artificial chromosome in yeast, in particular *S. cerevisiae*. Because the transferred DNA carries marker sequences from the vector, it is also possible to follow the transformation of the coccidia by detecting such sequences in genomic DNA of oocysts recovered after passage by use of conventional DNA-DNA hybridization methods or a sequence amplification method (DNA polymerase chain reaction, or PCR).

The packaged DNA is physically transferred into the recipient host organism by typical methods such as electroporation, microinjection or by *in vitro* incubation

of the DNA at high ionic concentration with the free living stages of the organism. Preferably, the transfer of the DNA is accomplished by electroporation or microinjection, most preferably by electroporation.

Electroporation is a simple and rapid method of introducing foreign DNA into a cell line. Cells are subjected to a short electric discharge, apparently producing reversible pores in the cell membrane. DNA is then capable of passing into the cell where it may be incorporated into the genetic library.

In order to increase the selectivity of the recombinant coccidia, one or more selectable markers are preferably used. For example, the donor organism for the DNA preferrably encodes for a selectable marker, such as, for example, drug resistance while the recipient organism is devoid of the marker such as, for example, being sensitive to the drug. After production of the recombinant coccidium, the recombinant coccidium is passaged in a suitable host and the selectable marker is utilized to permit replication of only the recombinant organism. For example, if drug resistance is utilized as the selectable marker, the host animal is administered the drug to which the donor organism is resistant during passage of the recombinant organism. In this way only those recombinant organisms which carry the drug resistance marker from the donor DNA will replicate and reproduce.

The present invention is particularly suitable for use with coccidia of the genera *Eimeria* which infect poultry hosts. The DNA from donor organisms of one or more particularly pathogenic species is introduced into recipient host organisms of a less pathogenic species to produce a recombinant organism which will exhibit the less pathogenic infectivity of the host recipient organism while expressing the antigenic character of the more pathogenic donor species. In this way, the recombinant organism will

enable the development of a safer vaccine to immunize the animal against the potentially lethal species of *Eimeria*. For example, the DNA from *E.tenella* and/or *E.necatrix* which are the most pathogenic species of *Eimeria* to infect the chicken is incorporated into a less pathogenic species such as *E.acervulina*. Similarly, for the turkey the DNA from for example *E. adenoides* could be incorporated into a less pathogenic species of *Eimeria* which infects turkeys. For the purpose of the detailed examples following, the chicken model utilizing *E. acervulina* as the recipient host organism and *E. tenella* as the donor of the DNA was utilized. It will be appreciated by those of skill in the art that the methodology utilized in the examples is applicable to other systems, namely other species of *Eimeria* which infect chicken, other species of *Eimeria* which infect other hosts, and other species of coccidia in other hosts.

E. acervulina, the most prolific species of chicken coccidia, is only mildly pathogenic, causes maladsorption in the duodenum and is rarely, if ever, fatal. It evokes protective immunity with greater ease than *E. tenella* and has a shorter prepatent period of 5 days versus 7 days for *E. tenella*. This gives an apparent advantage in that, with respect to the incorporated genes of *E. tenella* while being able to express antigenic determinants, the genes would not have sufficient time to encode for a complete *E. tenella* life cycle.

In terms of molecular biology, haploid *E. acervulina* has a DNA content comparable to that of *E. tenella* (7.25×10^{-15} g/nucleus or about 69,000 kb) (Cornelissen et al 1984). This amount of DNA is about 17 times that of *E. coli* (4,000 kb), an organism used by Clarke et al (1987), Danforth and Augustine (1986) and Murray and Galuska (1987) to produce antigens. Incorporating the *E. tenella* genes into *E. acervulina* not only results in a biological producer of the desired

antigens, but also results in a natural vector for use in a suitable vaccine. This approach circumvents the problems inherent in methods used by others where monoclonal antibodies and complementary DNAs have to be synthesized, purified and then used to produce antigens in unrelated organisms, before assembly into a subunit vaccine. This vaccine, if and when available, would still need to be injected or introduced into individual chickens.

By suitable techniques, the whole or part of the DNA from the nucleus of unsporulated *E. tenella* is isolated and inserted into unsporulated oocysts or sporozoites of *E. acervulina*. Recombinant *E. acervulina* oocysts are then sporulated and the sporulated oocysts and/or sporozoites enriched by feeding them to coccidia-free days old chicks and the feces collected after a suitable patency period. The presence of *E. tenella* DNA in the collected oocysts is confirmed and the progeny utilized to immunize birds which are then challenged with *E. tenella* as well as *E. acervulina*.

A method for isolating genomic DNA from oocysts of *Eimeria* species was developed utilizing a French press and phenol and ethanol extractions. Such DNA appears to be very "crude"; it is sheared (<100 kbp in size), and it has variable amounts of contaminants (nuclease activity, polysaccharides and membraneous material) associated with it. This crude DNA responds to exogenously added, purified restriction endonucleases but does not yield very many discrete fragments like yeast DNA which is similar in genomic complexity. Not much evidence for repeat DNA sequence was seen using usual methods. This suggests that the DNA is of low quality but that it was still useable in testing DNA transfer methods.

An existing vector containing protozoan teleomeric sequences and which functions as an artificial chromosome in yeast (YAC) was selected for packaging the

donor DNA for transfer of the donor DNA into the recipient organism. A recent vector pYAC-RC (provided by Douglas Marchuk and Francis Collins, used in Cystic Fibrosis gene mapping), was utilized.

Since YAC vectors use telomeric sequences from a protozoan, such sequences are likely to also work in *Emeria*. Upon removal of a segment of the p-YAC-RC vector DNA by cutting with BamHI nuclease (p-YAC-RC is a circular DNA plasmid), DNA sequences that function as telomeres (ends of chromosomes) are exposed. The remaining large DNA fragment is further cut with SmaI nuclease to generate two "arms". *E. tenella* DNA cut with SmaI nuclease is combined with the vector fragments and ligated to create "chromosomal-like" fragments with one or more of the "arms". On introducing these hybrid DNAs into *E. acervulina* oocysts, such sequences replicate alone or with host chromosomal DNA via integration during subsequent stages of chicken infection. Because the transferred DNA carries YAC sequence it is possible to detect/identify such sequences in genomic DNA of oocysts recovered after passage through a chicken by use of conventional DNA-DNA hybridization methods or a sequence amplification method (DNA polymerase chain reaction or PCR).

Construction of vectors containing the donor DNA employs standard ligation techniques. Isolated DNA fragments are cleaved, tailored and religated in the form desired to form the vectors required.

Cleavage or digestion is performed by treating with restriction enzyme (or enzymes) in suitable buffer. In general, about 1µg of DNA is used with about 1 unit of enzymes and about 20µL of buffer solution. Appropriate buffers and substrate amounts for particular restriction enzymes are generally specified by the manufacturer of the enzyme. Owing to the size of the DNA from the coccidia incubation times of about 4 hours to 24 hours at 37°C are

preferable. After incubation protein is removed by extraction with phenol and chloroform and nucleic acids recovered from the aqueous fraction by precipitation with ethanol.

Ligation refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments. For ligation, approximately equal molar amounts of the desired components suitably end tailored to provide correct matching are treated with about 10 units T4 DNA ligase per 0.5µg of DNA. When cleaved vectors are used, it may by useful to prevent religation of the cleaved vector by pre-treatment of vector DNA with alkaline phosphatase.

Southern analysis is a method by which the presence of DNA sequences in a digest of DNA containing compositions is confirmed by hybridization to a known labelled oligonucleotide or DNA fragment. For the purposes herein unless otherwise provided, southern analysis shall mean separation of digest on a 0.8% agarose gel and transfer to nitrocellulose by the method of E. Southern, (1975) J. Mol Biol 98:503-517 and hybridization as described by T. Maniacus et al (1970) Cell 15 687-701

Transformation means introducing DNA into an organism so that the DNA is replicable either as an extra chromosomal elemental or a chromosomal integrant.

The following example is given to illustrate the invention, but the invention is not to be limited thereto.

ISOLATION OF *EIMERIA* OOCYSTS AND SPOROZOITES

Unsporulated *E. tenella* and *E. acervulina* oocysts were isolated from fecal droppings of infected chickens by flotation in saturated NaCl. If desired, the

oocysts are allowed to sporulate by incubation in the salt solution for 18 to 20 hours at 30°C. The oocysts were surface-sterilized and decoated by washing in distilled water followed by treatment with 6% sodium hypochlorite for 10 min at 4°C. The decoated oocysts were washed thoroughly by centrifugation at 125 g for 1 min periods followed by resuspension. The washings were repeated at least 5 times in 0.9% NaCl and twice in homogenizing buffer containing 0.25 M sucrose and 0.1 M EDTA in 0.01 M Tris-HCl buffer pH 7.0 with added penicillin (100 units/ml) and streptomycin (50 µg/ml). The washed oocysts were centrifuged at 1,500 g for 10 min and the pellets resuspended and counted.

Sporocysts were released by fracturing the oocyst walls using unsiliconized glass beads. Oocysts were concentrated to approximately $8x10^6$ oocysts/ml. 10.5g of 450-500µ glass beads per 2.5ml of oocyst suspension is placed in a one-ounce universal bottle and vortexed on high for 15 seconds. The beads were rinsed in sterile water and gravity filtered through a glass bead funnel in order to remove most of the wall debris and unbroken oocysts. Glass bead funnels were prepared by filling 150ml sintered glass Buchner funnels with 200µ unsiliconized glass beads up to a height of 5cm and rinsing once with filtered water before loading with the smashed oocyst solution. Sporocysts were rinsed through the glass beads until oocysts are visible in the filtrate.

After filtering the sporocysts, they were centrifuged and resuspended at $10x10^6$ oocyst/10ml of medium-199. (Gibco Instant Tissue Culture Medium pH adjusted to 7.5) Sporocysts were then incubated at 41°C for 1/2 hour in Difco Bacto Bile Salts at a concentration of 50mg/10ml of solution followed by Trypsin at 10mg/ml or solution and incubated at 41C for the additional one hour to release sporozoites. *E. acervulina* sporozoites are fragile relative to other stages in the *Eimerian* life cycle and should be kept on ice immediately after excystration

and should be subjected to minimal centrifugation to retain viability.

Glass beads were rinsed once with Ringer's solution before sporozoite/bile/trypsin suspension were loaded onto the glass bead funnels and sporozoites passed through without the aid of suction. If significant residue blocks the filter, the surface beads were stirred using a wooden stirring rod. Sporozoites were then rinsed through until the effluent contained increased percentages (greater than 1%) of sporulated oocysts. The sporozoites were purified by passing them through a second, clean glass bead filter using the same method described above. Sporozoites were then collected by centrifugation at medium speed for 3 minutes and resuspended in Ringer's solution. Recovery of sporzoites was usually 70% or better with purities greater than 98.5%.

Glass beads were cleaned by soaking them in nitric acid followed by extensive rinsing in tap water. Nitric acid is removed from the beads by stirring them in distilled water until the pH of the distilled water added remained constant.

PREPARATION OF DONOR DNA

E. tenella oocysts were collected and washed in Javex as set out above. They were then washed 3 times in 20mM Tris buffer (pH adjusted to 7.5) and 10mM EDTA solution before smashing in the French press. Cells were concentrated and resuspended in approximately 10ml of Tris/EDTA solution and passed once through the French press at 10-12 tonnes. Sodium dodecal sulfate (SDS) was added to a final concentration of 1% in order to denature protein. Caution must be taken not to exceed 1% SDS since the DNA may precipitate and centrifugation should be done at room temperature.

Proteinase K was added to a concentration of 100µg/ml and the pH was adjusted to 8 using a concentrated Tris base. Digestion of protein was allowed to proceed overnight at 37°C. DNA was extracted with an equivolume of phenol and inverted gently (taking care not to shear DNA) for 5 minutes doing all extraction in the fumehood and the centrifuged at room temperature 10-15 minutes at 5000rpm. If the top layer was not clear, the top layer was removed, reextracted with, phenol and centrifuged.

The top layer was pipetted off and extracted with equivolume of 50:50 phenol: half chloroform, inverted gently for 5 minutes and centrifuged for 10-15 minutes at 5000 rpm. The top layer was removed, extracted with equivolume of chloroform, inverted gently for 3-5 minutes and centrifuged for 10-15 minutes at 5000 rpm. The supernatant was removed and the molarity increased to 0.3M by addition of 1/10 volume of 3M sodium acetate. (pH of 5.2) Two volumes of absolute ethanol or 1 volume isopropanol was added quickly in order to precipitate DNA.

The precipitated DNA was centrifuge at 3000 rpm to form a pellet. The ethanol was carefully poured off so as not to drain off the soft pellet. DNA was solubilized in a minimum TE solution ensuring that all DNA has solubilized as some may remain as a clear gel. Pre-boiled RNase was added up to 50µg/ml and before incubation at 37C for 1 hour a small aliquot was measured and its absorption level at 260nm recorded.

In order to remove RNase proteinase K was added up to 200µg/ml and incubated at 37C for 1 hour. To purify DNA phenol, phenol/chloroform(1:1), and chloroform were added respectively at an equivolume. After each successive addition the extracts were inverted gently for 5 minutes, centrifuge 10-15 minutes at 5000 rpm and the bottom layer discarded.

After the last centrifugation was complete, the top layer was removed and the solution was placed into a dialysis bag and dialyzed in a sterile 1L beaker of TE solution overnight in order to reduce nucleotides in the solution. The TE solution was replaced and allowed to sit for an additional 1 to 2 hours. A spectometer reading at 260nm of the TE solution should approach 0 as fewer nucleotides are being removed from the dialysed DNA solution.

After dialysis spectrometer readings were taken at wavelengths of 260nm and 280nm. One OD unit at 260nm is equivalue to 50µg/ml of DNA. A ratio of OD units from readings at wavelengths 260nm and 280nm indicate the purity of the sample. Ratios between 1.6 and 1.8 indicate pure DNA whereas ratios of 2 and upwards indicate significant residue present.

Digestion and ligation of DNA

Three samples of *E.tenella* DNA (150 µg in 250 µl final volume of digestion buffer) were digested for 0.5, 2, and 4 hr respectively at 37°C with 10 units of SmaI endonuclease (cuts at sequence: 5'-NNCCC< > GGGNN-3'). 15 µg of YAC vector (plasmid pYAC-RC) was digested under the same conditions with 1 unit each of BamHI and SmaI. After stopping the respective reactions with 0.1% SDS and 25mM EDTA at 70°C for 5 min, all of the samples were pooled and extracted twice with 1 volume of phenol-chloroform. DNA was precipitated with 2.5 volumes of 95% ethanol (v/v) and centrifugation (Beckman 12 microfuge). DNA was semi-dried with N_2 gas and dissolved in 250µl of 1mM Dithothreitol in H_2O at 36°C for 24 h before addition of 5X ligation buffer (66mM Tris-HCl pH 7.8, 6mM $MgCl_2$ final conc.), freshly made ATP (0.5mM final) and 30 µl (60 units) of T4 ligase (Pharmacia). Ligation of *E.tenella* DNA to plasmid vector

DNA was carried out for 30 hr at 5°C, before further dilution with 1X ligation buffer without ligase to approx. 500µl final volume. Ligated DNA was subsequently stored at 4°C until used.

Electroporation of *E. acervulina*

Sporozoites were prepared as described earlier. The cell-porator unit supplied by Bethesda Research Laboratories Life Technologies, Inc. in conjunction with a cell-porator voltage were used containing two electrodes 4cm apart, 1 ml of sporozoite suspension at concentrations greater than $1x10^6$/ml was loaded into the chamber using a micro pipette. Although cell fusions may occur at such high concentrations, a greater percent of transformation is thought to occur when cell concentrations exceed $1x10^6$/ml. DNA was added to each cell suspension at a concentration not less than 20µg/ml. Chambers were kept on ice while cells were subjected to the electrical pulses at 400V, 330µF capacitance, 100kV booster and high resistance. The sporozoite suspensions were thereafter allowed to remain on the lab bench for at least 10 minutes in order to enhance DNA uptake.

Several trials were carried out using different stages of the Eimerian life cycle, sporocysts and oocysts. As well some sporozoite and oocyst suspensions containing acridine orange in addition to the DNA were studied as acridine orange may increase DNA uptake.

Inoculation of chicks with Recombinant *E. acervulina*

Groups of days old chicks were inoculated per os with about 500,000 sporozoites each of untreated *E.acervulina*, *E.acervulina* into which had been tranferred *Clostridium* sp. DNA, *E.acervulina* into which had been transferred *E. tenella* DNA and *E.acervulina* into which had been transferred acridine orange labelled *E. tenella DNA*.

Thirteen days after inoculation each group was challenged blind with 50,000 *E. tenella* oocysts per bird. Six days after challenge, each bird was scored blind for lesion size and the results tabulated.

ANALYSIS OF DNA

Preparation of DNA Samples

i) Genomic DNA- Approx. 50 ug of DNA from *E.tenella*, *E.acervulina* and the two putative *E.acervulina* x *E. tenella* recombinants isolated as desribed above were each incubated with 50 units of BamHl enzyme (cuts at sequence: 5'NNG< >GATCCNN-3') at 37°C for 4 hr and 24 hr in buffer containing 10mM Tris.HCl pH 7.8, 50mM KCl, 0.1mM EDTA, 1mM Dithiothreitol. Reactions were stopped by addition of sodium dodecylsulphate (0.2% final) and EDTA (25mM).

ii) Marker DNA: plasmid pYAC-RC (5 µg) and lambda bacteriophage, Charon 4A (5µg) were digested as described for genomic DNA (see above) with 1 unit each of BamHl, HindIII and Eco Rl.

b) Electrophoresis, DNA Transfer to Membranes and Hybridization.

DNA samples were loaded into slots (lanes in a 15cm x 20cm, 0.8% (w/v) agarose slab containing Tris-Acetate, EDTA, ethidium bromide (5ug/ml) buffer and electrophoresed (submerged in same buffer without ethidium bromide) for 6hrs at 40mA, 18°C. DNA in gel was photographed (Polaroid type 52 film using UV light and filter). Gel was further treated with 100mM HCl at 20°C for 10 min. and 400mM NaOH for 30 min to break and denature the DNA strands. After three brief washes in gel running buffer, DNA in the gel was electrophoretically transferred to Hybond-N (Amersham)

0.45 µm nylon membrane (same size as gel). The membrane was rinsed in a solution containing 1M NaCl and 1mM EDTA and dried by air (30min) and heat (30°C, 1 hr in vaccuo). The membrane was incubated for 1 hr at 68°C in 200 ml of hybridization buffer (1M NaCl, 1mM EDTA, 0.1% SDS, 10µg/ml sheared and dematured calf thymus DNA (Sigma Chem). Hybridization was carried out in a plastic bag containing 10 ml of same buffer stock with 10^7 cpm of random primed pYAC-RC (0.5 ug) template. Membrane was washed four times with 100ml of hybridization buffer at 1X and four times with 100ml of hybridization buffer at 0.5X at 68°C. Filter was air dried and exposed to Kodak XR X-ray film with single screen for three days and 5 days.

RESULT AND DISCUSSION

Differences in samples of DNA were noted during processing of *E. acervulina* and *F. tenella* DNA. After French pressing, the *E. acervulina* solution seemed very gel-like, whereas the viscosity of the *E. tenella* did not visibly change. Upon phenol addition, *E. acervulina* DNA formed a very tight white precipitate at the interface whereas *E. tenella* precipitate breaks up at the interface. After addition of ethanol, *E. tenella* turns the DNA suspension cloudy whereas the *E. acervulina* suspension remains clear and DNA is visible as a fluffy cotton ball type precipitate. *E. tenella* DNA precipitate after freezing is a fine powder. In general the DNA samples prepared from the first generation electroporated oocysts appeared as the unaltered *E. acervulina* DNA however, after precipitation with ethanol some of the precipitate floated and some separated and sank to the bottom of the tube. This may be an indication of an alteration in the *E. acervulina* DNA.

ANALYSIS OF DNA BY ELECTROPHORESIS

Fig. 1A shows a photograph of ethidium bromide stained, electrophoretically separated DNA in agarose gel before transfer of DNA to nylon membrane. Fig 1B shows a photograph of X-ray film indicating distribution of radioactive YAC probe sequence bound to DNA on the nylon filter.

Lanes a and m, negative control (does not hybridize to YAC probe as expected) and DNA size markers: lambda bacteriophage Charon 4A DNA digested with BamH1, HindIII and EcoR1. Charon 4A digest should yield 11 fragments but only six large fragments 4.4-11.5 kbp in size (see six bars right side of photo) were observed.

Lane h, BamH1+ HindIII + SmaI digested PYAC-RC (positive control) should yield at least eight ethidium bromide staining bands (more with partial digestion as appears to be the case as SmaI does not cut well). Probe made from this DNA hybridizes to all fragments (Fig. 1B).

Lanes b-f and lanes i-l correspond to *Emeria* sp. DNA digested with BamH1 (see Experimental for details). Lane g, DNA from a *Clostridium* sp. also isolated from chicken faeces (serves as another negative hybridization control). *E.tenella* DNA (lanes b and c), *E. acervulina* (lanes d, e) and putative *E. acervulina* x *E.tenella* recombinants first trial (lanes i, j) and second trial (k and l). Lanes b, d, g, j and k are DNA samples digested for 4 hrs at 37°C. Lanes c, e, f, i, and l are samples of DNA digested with same amount of BamH1 enzyme at 37°C but for 24hrs.

b) Interpretations:

Fig. 1A indicates that all DNA samples have endogenous nucleases (compare 4 hrs vs. 24 hr digests). Other tests indicated that this nuclease activity is

inhibited mostly by EDTA, requiring $MgCl_2$ which is present in the restriction enzyme buffer. There is also evidence that the "crude" DNA samples contain (see ethidium bromide staining material particularly at top of gel) cellular debris rich in polysaccharide and lipid. This material is entangled with high molecular DNA and is released from extensively degraded DNA (24hr samples); most of it floated out of the gel wells during electrophoresis, the rest escaped during handling of the gel for photography. These observation are very typical of crude DNA preps of most lower organisms.

No YAC probe hybridization to lamda Charon 4A in spite of very high concentration of this DNA which is a very good control for probe specificity, suggesting that hybridization to extra bands in lane j may be real. No hybridization to *Clostridium* sp DNA observed also is another good sign of specificity.

No hybridization to low molecular weight DNA of *Emeria* parental DNAs (4hr digests); slightly more to *E.tenella* than *E.acervulina* DNA. The ethidium bromide staining patterns (Fig1A) of *Eimeria* parental DNA show traces of repeat sequences which do not hybridize to YAC probe. However the *E.acervulina* recombinant # 1 DNA which gave the lowest lesion score (1.2) has a pattern of hybridization a bit like *E.tenella* DNA but it also has two positive hybridization bands, one being similar in size to YAC plasmid DNA used originally in the ligations. In late digestion (24hr) all of this hybridization material is likely lost as might be expected by extensive endogenous nuclease nicking of the DNA.

Recombinant # 2 DNA also has a small amount of hybridization in the same position but the concentration is much lower. Since these samples represents pools of oocysts this could mean that #1 has been amplified more than #2.

The positive bands in lane j tend to indicate the presence of true YAC sequence, but not all of the sequence. This would be the simplest and most consistent interpretation of the data and would not be surprising because the specific *E.tenella* DNA or *E.acervulina* DNA associated with it would could have fused and discarded either all or part of the YAC arms through sequences related to the Trp1, Ura3, TEL or His3 gene sequences. The yeast genes are conserved in lower eukaryotes and so possibly is the TEL gene.

The observed bands may be related to *Eimeria* repeat sequences which would require highly intact DNA t- confirm but the control data do not strongly support this possibility. The possibility of spurious hybridization is also unlikely due to predictability of results from the other controls. Also the same trend was continued when hybridizations were repeated on a more limited scale by dot method due to lack of sample.

Results of immunization studies

Groups of days old chicks were inoculated with samples of each of untreated *E. acervulina*, *E. acervulina* in to which have been transferred *Clostridium sp.* DNA, *E. acervulina* in which have been transferred *E. tenella* DNA, and *E. acervulina* in to which have been transferred acridine orange labelled *E. tenella* DNA. Thirteen days after inoculation the chicks were challenged with 50,000 *E. tenella* oocysts and after suitable patency period, birds were explored for lesion size. Summary results of a number of tests are given in the following Tables 1 and 2.

TABLE 1

Group	Ave Lesion Score	Reduced Lesion/Total
Control	3.0	0/8
Clostridium DNA	2.7	2/14
E. tenella DNA	2.8	3/13
Labelled *E. tenella* DNA trial#1	1.5	6/7
trial#2	1.2	

TABLE 2

Group	Average Lesion Score
Not porated Control	3.0
Porated with *Clostridium* DNA	3.3
Porated with *E. tenella* DNA	2.8
Porated with labelled *E. tenella* DNA trial #1	1.5
trial #2	1.2
Not porated Sporozoites	2.6
Not porated Oocysts	3.3

Porated with *Clostridium* DNA	2.1
Porated with *E. tenella* DNA	2.7

The above results clearly demonstrate the effectiveness of the transfered DNA of the *E. tenella* donor to confer immunity to *E. tenella* in birds immunized with the recombinant *E. acervulina*. This is evidenced by the reduced lesion scores in those animals inoculated with the recombinant organism. The reduction in lesion scores seen with the *Clostridium* sp. was postulated to arise as a result of possibility of cross contamination between the birds inoculated with the two recombinant organisms. It is also clear from the results that, at least under the conditions selected for the electroporation, the use of the acridine orange label increases significantly the efficiency of the transfer of the donor DNA to the recipient host. The average lesion scores of 1.5 and 1.2 and the degree of protection with 6 out of 7 birds having significantly reduced lesions are similar to results that are normally observed upon challenge of birds inoculated with native *E. tenella* in traditional immunization programs.

The above example clearly demonstrates the ability of the recombinant coccidia in the present invention to confer immunity in an animal host against infection by the coccidium which acts as a donor organism. While the example has only utilized whole DNA from one species it would be readily apparent to those of skill in the art that the invention could be practiced utilizing more than one species as a donor organism thereby conferring immunity to multiple specie of coccidia in an animal host. Also as the understanding of coccidial antigenic determinants and the immunity evoked in the host animal by immunization with a coccidia vaccine increases in

the future the invention may be utilized to take advantage of this increased knowledge. For example, as significant antigens and the genes encoding them are identified, such genes may be isolated and packaged in accordance with the present invention to permit the expression of the gene and production of the antigenic determinant in the recombinant coccidium. Such genes could include genes from many species of coccidia thereby conferring immunity to a multitude of species through the use of the recombinant coccidium of the present invention.

The recombinant coccidia of the present invention permits the development of novel vaccines which induce immunity to more pathogenic species of coccidia by incorporating the DNA from such species into less pathogenic species. Such a vaccine has a major impact on the concept of coccidial vaccine manufacturing. The vaccine will be used with less impunity than present live vaccines and the industry now has a viable and longer-lasting alternative to medication. By utilizing a less pathogenic species of coccidia as the recipient, the potential for accidentally inducing disease in a flock through misadministration of the vaccine is greatly reduced. Vaccine doses can be increased relative to traditional vaccines to ensure that even if individual chicks receive reduced doses, there will be sufficient antigen present to confer immunity. Unlike present live vaccines, the vaccine of the present invention does not require anticoccidials either in the feed or the water to assist in control of coccidiosis.

Although various preferred embodiments of the present invention have been described herein in detail, it will be appreciated by those skilled in the art, that variations may be made thereto without departing from the spirit of the invention or the scope of the appended claims.

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THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE AS FOLLOWS:

1. A recombinant coccidium comprising a relatively non-pathogenic strain of coccidia as an recipient host containing DNA from one or more donor coccidia selected form other strains or species of coccidia wherein the recombinant coccidium expresses surface antigenic determinants encoded by the DNA of the donor organism.

2. A recombinant coccidium as claimed in Claim 1 wherein the host and donor are from the genus *Eimeria*.

3. A recombinant coccidium as claimed in Claim 2 wherein the host coccidium is *E. acervulina*.

4. A recombinant coccidia as claimed in Claim 3 wherein the donor coccidium is selected from one or more of *E. tenella*, *E. neaotrix*, *E. brunetti* and *E. maxima*.

5. A recombinant coccidia as claimed in Claim 4 wherein the donor coccidia is *E. tenella*.

6. A method of producing a recombinant coccidium comprising:

a) extracting DNA from a donor coccidium, and

b) introducing the extracted donor DNA into an recipient host coccidium in a manner to permit expression of the donor DNA in the host coccidium.

7. A method as claimed in Claim 6 wherein the donor DNA is packaged into a vector containing telomere DNA sequences.

8. A method as claimed in Claim 7 wherein the vector functions as a yeast artificial chromosome (YAC).

9. A method as claimed in Claim 8 wherein the YAC is pYAC-RC.

10. A method as claimed in Claim 6 wherein the donor DNA is introduced into the recepient host coccidia by electroporation, microinjection or incubation in high ionic strength buffer.

11. A method as claimed in Claim 10 wherein the donor DNA is introduced into the recipient host coccidia by electroporation.

12. A recombinant coccidiosis vaccine comprising a relatively non-pathagenic recepient host of coccidia to which has been introduced sufficient DNA from one or more coccidia selected from donor other strains or species of coccidia to produce immunity to the recipient and donor strains in a host animal through inoculation of the host animal by the recombinant coccidia.

13. A recombinant coccidiosis vaccine as claimed in Claim 12 wherein the host and donor are from the genus *Eimeria*.

14. A recombinant coccidiosis vaccine as claimed in Claim 13 wherein the host coccidia is *E. acervulina*.

15. A recombinant coccidiosis vaccine as claimed in Claim 14 wherein the donor coccidia is selected from one or more of *E. tenella*, *E. necatrix*, *E. brunetti* and *E. maxima*.

16. A recombinant coccidiosis vaccine as claimed in Claim 15 wherein the donor coccidia is *E. tenella*.

17. A recombinant coccidiosis vaccine according to any one of Claims 12 to 17 wherein the recombinant coccidia is diluted in an aqueous edible gum solution which results in uniform suspension of said coccidia.

18. A method of immunizing an animal against infection by coccidia comprising inoculating the animal with a recombinant coccidiosis vaccine comprising a relatively non-pathagenic recipient strain of coccidia to which has been introduced sufficient DNA from one or more coccidia selected from donor other strains or species of coccidia to produce immunity to the recipient and donor strains in a host animal through inoculation of the host animal by the recombinant coccidia.

Figure 1